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Microreview

Toxin entry: how bacterial proteins get into mammalian cells

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Summary

Certain bacteria secrete protein toxins that catalytically modify and disrupt essential processes in mammalian cells, often leading to cell death. As the substrates modified by these toxins are located in the mammalian cell cytosol, a catalytically active toxin polypeptide must reach this compartment in order to act. The toxins bind to receptors on the surface of susceptible cells and enter them by endocytic uptake. Endocytosed toxins initially accumulate in endosomes, where some of these proteins take advantage of the acidic environment within these organelles to form, or contribute to the formation of, protein-conducting channels through which the catalytic polypeptide is able to translocate into the cytosol. Other toxins are unable to respond to low pH in this way and must undergo intracellular vesicular transport to reach a compartment where pre-existing protein-conducting channels occur and can be exploited for membrane translocation – the endoplasmic reticulum. In this way, cell entry by this second group of toxins demonstrates that the secretory pathway of mammalian cells is completely reversible.

Introduction

Many bacteria produce and secrete protein toxins that are important virulence factors in a range of human diseases. With the exception of pore-forming protein toxins, which compromise the permeability barrier of the target cell plasma membrane, the bacterial toxins act catalytically to modify substrates within mammalian cells. These substrates normally play key roles in essential cellular processes, such as signal transduction, cytoskeleton assembly, vesicular trafficking or protein synthesis. Because the substrates modified are all located in the cytosol, a catalytically active toxin polypeptide has to enter this compartment, a step that entails translocation of the polypeptide across a membrane. In this review, we outline current concepts to explain the entry process for protein toxins that exploit the normal mechanisms used by mammalian cells during the uptake of material from the extracellular environment and pre-existing intracellular protein trafficking pathways. Recently, increased attention has been focused on the site and mechanism of membrane translocation during the process of cell entry. It is now clear that entry into the cytosol can occur in one of two ways: translocation from endosomes by a mechanism that requires low pH, or translocation from the lumen of the endoplasmic reticulum (ER) by a mechanism that uses protein-conducting channels present in the ER membrane. To illustrate these two entry mechanisms in this review, we focus on the subset of protein toxins that act by inhibiting cellular protein synthesis, as members of this subset are known to use both translocation mechanisms. The other catalytic toxins enter cells in the same manner, and reference will be made to them where appropriate. It should be noted that certain bacteria have evolved alternative mechanisms for delivering their proteins into mammalian cells. These include type III secretion used by several Gram-negative bacteria to deliver material into target cells via the needle complex, a 'molecular syringe' that injects bacterial factors into the host (Galan and Collmer, 1999). Other pathogens, such as *Shigella* and *Listeria*, enter macrophages and related cells by phagocytotic uptake, before the bacteria themselves or their putative toxins enter the cytosol by lysing, or making pores in, the phagosomal membrane (High *et al.*, 1992). These alternative cell entry mechanisms are not considered further here.

Toxins that inhibit eukaryotic protein synthesis

Toxins that modify substrates essential for cellular protein synthesis include diphtheria toxin (DT) from *Corynebacterium diphtheriae*, exotoxin A (PE) from *Pseudomonas aeruginosa*, shiga toxin (ST) from *Shigella dysenteriae* and the shiga-like toxins (SLTs; sometimes called verotoxins) produced by a number of enterohaemorrhagic *Escherichia*

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coli (EHEC) serotypes. DT is produced by strains of *C. diphtheriae* that have been infected by a specific bacteriophage known as the β-phage. It is the major virulence factor in diphtheria, an acute, contagious, febrile illness. During infection, *C. diphtheriae* grows in the upper respiratory tract, where the DT secreted causes necrosis and inflammation of the mucosa. *P. aeruginosa* infection is prevalent among patients with burns, cystic fibrosis or acute leukaemia, whereas *S. dysenteriae* is the infectious agent responsible for epidemics of bacillary dysentery. The *E. coli* serotypes that produce the SLTs, such as *E. coli* O157:H7, cause haemorrhagic colitis and haemolytic uraemic syndrome.

DT and PE are synthesized and secreted by the producing bacteria as single-chain polypeptide precursors but, in their functional form, they exist as heterodimers in which both polypeptides are covalently joined by a single disulphide bond. The DT and PE precursors are cleaved into dimers after entering mammalian cells by the ubiquitous protease furin (Gordon *et al.*, 1995). In the heterodimeric toxins, one polypeptide (designated the A chain or fragment) is catalytically active, while the other (the B chain or fragment) is responsible for binding the heterodimer to receptors present on the surface of susceptible cells. In ST and the SLTs, the A chain is non-covalently associated with a pentamer of B chains (Fraser *et al.*, 1994), the so-called AB_5 structure characteristic of the cytotonic toxins, cholera toxin (CT) (Merritt *et al.*, 1994) and *E. coli* heat-labile enterotoxin (Sixma *et al.*, 1991). The A chains of ST and the SLTs contain two cysteines joined by a disulphide bond, and the loop between these two residues contains a furin cleavage site. Cleavage at this site during cell entry converts the 32 kDa A chain into a catalytically active 27.5 kDa A_1 fragment and a 4.5 kDa A_2 fragment (Garred *et al.*, 1995) that interacts with the B pentamer (Fraser *et al.*, 1994).

DT and PE inhibit protein synthesis by catalysing the ADP-ribosylation of elongation factor 2 (EF-2; van Ness *et al.*, 1980). The ADP-ribose moiety from NAD^+ is added to a modified histidine residue, known as diphthamide, uniquely present in EF-2. In contrast, ST and the SLTs are RNA-specific N-glycosidases that catalyse the removal of a specific adenine residue (A-4324 in rat liver 28S rRNA) from the large RNA component of eukaryotic ribosomes, the same mode of action as that used by the related plant toxin ricin (Endo *et al.*, 1987). Ribosomes containing toxin-depurinated 28S RNA are unable to bind elongation factors and are therefore incapable of protein synthesis. To reach their cytosolic substrates, the toxins must bind to the target cell surface. Binding can be to specific proteins or lipids: DT binds to a heparin-binding epidermal growth factor-like growth factor precursor (Naglich *et al.*, 1992), PE to the α_2-macroglobulin receptor (Kounnas *et al.*, 1992), while ST and the SLTs bind to globotriasoylceramides, a subset of glycosphingolipids (Jacewiez *et al.*, 1986).

Toxin entry into cells

Surface-bound toxin enters cells by endocytosis, the endocytic route used being the one normally followed by the receptor. Certain toxin–receptor complexes (for example, DT and its receptor) enter via plasma membrane-derived vesicles whose cytoplasmic surface is coated with the protein clathrin. Because of this, the overexpression of a *trans*-dominant negative mutant of the GTPase dynamin, which blocks clathrin-mediated endocytosis, protects cells from intoxication by DT (Simpson *et al.*, 1998). Other toxins enter cells by clathrin-independent endocytosis: CT, which binds to the ganglioside G_{M1}, enters through cholesterol-rich plasma membrane domains called caveolae (Orlandi and Fishman, 1998), and other clathrin-independent, non-caveolar endocytic routes are also used (Simpson *et al.*, 1998). ST and the SLTs also enter cells exclusively via clathrin-dependent endocytosis (Sandvig *et al.*, 1991a), suggesting that their glycolipid receptor becomes fixed in clathrin-coated surface pits, possibly by interacting with a protein already anchored there. Regardless of the endocytic carrier used for internalization, the toxins are all initially delivered to early endosomes.

Translocation from endosomes

Early endosomes are tubular/vesicular organelles distributed primarily in the peripheral cytoplasm. The internal pH of endosomes is acidic, the low pH being established and maintained by a membrane-associated, proton-pumping ATPase. The low pH environment is essential for certain normal sorting functions of endosomes, such as the dissociation of ligands from receptors during receptor-mediated endocytosis, and several bacterial toxins use it to their advantage to enter the cytosol (Fig. 1). Toxins whose A fragment crosses the early endosomal membrane include DT (Lemichez *et al.*, 1997), which has been the most thoroughly studied, and others such as anthrax, tetanus and botulinum toxins. Furin cleavage of DT generates a 21 kDa N-terminal A fragment and a 37 kDa C-terminal B fragment. The crystal structure of DT shows it to be a Y-shaped molecule containing three distinct domains, which account for the functional properties of the protein (Choe *et al.*, 1992). Fragment A is the catalytic domain, while fragment B actually consists of two domains. The C-terminal domain of fragment B contains 10 β-strands, which form two β-sheets and are responsible for receptor binding, whereas the middle domain (known as the transmembrane or T domain) contains nine helices, two pairs of which are unusually hydrophobic and facilitate the membrane translocation step. Upon exposure to the low pH in endosomes,

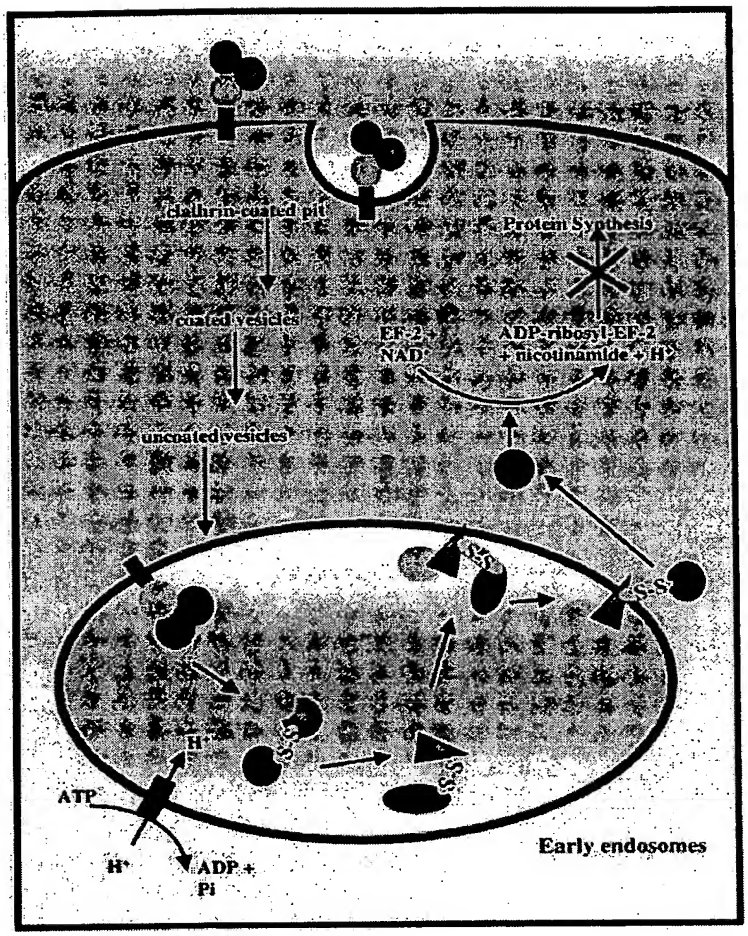

Fig. 1. Toxin entry into mammalian cells from acidified endosomes. DT binds to the cell via an interaction between its receptor-binding domain (yellow) and its surface receptor, and enters the cell by receptor-mediated endocytosis in clathrin-coated pits and vesicles. After uncoating, DT is delivered to early endosomes and is proteolytically cleaved by furin to generate the disulphide-linked, heterodimeric A-B form of the toxin. The low pH in endosomes generated by the proton-pumping ATPase probably causes the toxin to dissociate from its receptor and does cause all three toxin domains to form partially unfolded molten globule-like structures. Exposed hydrophobic helices in the transmembrane domain (purple) allow this domain to insert into the endosomal membrane to form, or form part of, a translocation channel through which the partially unfolded catalytic domain (red) is able to pass. In the cytosol, the free catalytic domain refolds into its biologically active conformation to inhibit protein synthesis by the irreversible ADP-ribosylation of EF-2, leading to the death of the cell.

DT undergoes a physiologically critical conformational change (Tortorella *et al.*, 1995). This change results in the partial unfolding of all three domains into molten globule-like structures. In the case of the T domain, this exposes the hydrophobic helices, allowing them to insert into the endosomal membrane where they form, or contribute to the formation of, a proteinaceous channel, which functions as a translocation pore for the partially unfolded A fragment (Draper and Simon, 1980; Sandvig and Olsnes, 1980; Zhan *et al.*, 1994). At present, the precise structure of the DT B-containing translocation pores that form in the endosomal membrane is not known. Exposure to endosomal pH therefore transforms the DT B fragment from a soluble, globular form to a transmembrane form. Once the translocated A fragment encounters the increased pH of the cytosol, it spontaneously refolds into its catalytically active native conformation. At some stage during the membrane translocation step, the disulphide bridge linking the DT A and B fragments is reductively cleaved (Papini *et al.*, 1993). Because DT A fragment translocation depends crucially on exposure to low pH, lowering the extracellular pH to that of endosomes causes the toxin to cross the plasma membrane directly (Sandvig and Olsnes, 1986), while treating cells with reagents that increase endosomal pH (Olsnes and Sandvig, 1991; Beaumelle *et al.*, 1992) or bafilomycin A, which inhibits the vacuolar H^+-ATPase (Umata *et al.*, 1990), protects against intoxication. Such treatments were without effect on the cytotoxicities of other bacterial toxins such as PE or ST, which do not depend on acidification to enter the cytosol. These toxins are unable to translocate from endosomes and must journey further into the cell before the compartment for translocation is reached.

Transport from endosomes to the endoplasmic reticulum

It is now clear that those toxins that are unable to respond

to low pH by forming a translocation channel in the endosomal membrane undergo retrograde vesicular transport via the Golgi complex to reach the lumen of the endoplasmic reticulum (ER), from where translocation into the cytosol takes place (Rapak *et al.*, 1997; Lord and Roberts, 1998). An early indication of this transport pathway came with the demonstration that treating cells with brefeldin A (BFA), a fungal metabolite that disorganizes the Golgi complex (Klausner *et al.*, 1992), protected them against intoxication by PE and ST (Yoshida *et al.*, 1991; Sandvig *et al.*, 1991b), but was without effect on sensitivity to DT (Yoshida *et al.*, 1991). BFA also protects cells against the effects of other bacterial toxins, including CT and pertussis toxin (Orlandi *et al.*, 1993; Baya *et al.*, 1997). More recently, direct biochemical evidence has been obtained for retrograde transport of toxins to the ER (Johannes *et al.*, 1997; Rapak *et al.*, 1997), and endocytosed ST and CT have been visualized within the ER lumen (Sandvig *et al.*, 1992; 1996). Certain proteins that recycle between the cell surface and the *trans*-Golgi network (TGN), such as the cation-independent mannose 6-phosphate receptor, are thought to do so via early endosomes and, subsequently, late endosomes (von Figura, 1991). In the case of ST, however, it has been shown recently that the toxin proceeds directly from early endosomes to the TGN without trafficking through late endosomes (Mallard *et al.*, 1998), a route that other toxins might also use.

Earlier work with PE had indicated that this toxin also undergoes transport from the TGN to the ER before translocating into the cytosol, and suggested a possible mechanism to account for this retrograde trafficking. The last five C-terminal residues of the translocated PE fragment are Arg-Glu-Asp-Leu-Lys (REDLK in the single-letter code). This sequence is similar to the tetrapeptide KDEL found at the C-terminus of resident proteins of the eukaryotic cell ER lumen, which is known to function as an ER retrieval signal (Munro and Pelham, 1987). Resident ER luminal proteins sometimes escape from the ER, along with newly synthesized secretory proteins, in ER-to-Golgi transport vesicles. While secretory proteins proceed through the Golgi stack *en route* to the cell surface, the ER-resident proteins are intercepted by a receptor protein, the KDEL receptor, distributed throughout the Golgi, including the TGN (Griffiths *et al.*, 1994), which interacts with them via their C-terminal KDEL sequence. The KDEL receptor–ligand complex then undergoes retrograde transport to return the protein to the ER lumen (Pelham, 1990). The effect of mutations in the REDLK pentapeptide of PE on its cytotoxicity to mammalian cells has been determined (Chaudhary *et al.*, 1990). While the terminal K residue could be deleted without effect, further deletions or substitutions predicted to compromise interaction with the KDEL receptor significantly reduced cytotoxicity (Chaudhary *et al.*, 1990). Such changes to the REDLK sequence did not affect the catalytic activity of PE or its ability to interact with and enter cells, suggesting that the reduction in cytotoxicity could have resulted from impaired transport to the translocation compartment – the ER lumen. The C-terminal Lys residue of the -REDLK sequence, which could prevent the interaction of PE with the KDEL receptor (Kreitman and Pastan, 1995), is removed by a carboxypeptidase during cellular intoxication (Hessler and Kreitman, 1997). Recently, it has been shown that the KDEL receptor does indeed function as a Golgi-to-ER carrier during the cellular entry of PE (Jackson *et al.*, 1999) (Fig. 2). CT and *E. coli* heat-labile enterotoxin contain KDEL and RDEL, respectively, at the C-terminus of the translocated polypeptide and, once again, the tetrapeptide is required for optimal activity of the toxins (Lencer *et al.*, 1995). Other toxins that are transported to the ER lumen, such as ST and the SLTs, clearly lack KDEL or a functionally related sequence on the translocated polypeptide. These proteins must therefore use a different, as yet unidentified, carrier for Golgi-to-ER transport (Jackson *et al.*, 1999).

Translocation across the endoplasmic reticulum membrane

At present, the mechanism by which protein toxins cross the ER membrane is unclear. It was initially proposed that toxins unable to make their own translocation channel in membranes must travel to a compartment where pre-existing protein-conducting channels are present and could be used (Pelham *et al.*, 1992). The ER membrane contains such channels whose central component is the protein Sec61, the Sec61p translocon. The Sec61p translocon normally functions in the import of nascent secretory proteins into the ER lumen (Rapoport *et al.*, 1996). It has also been shown that the Sec61 translocon can function in the reverse direction to export proteins from the ER lumen to the cytosol as part of the ER quality control function (Brodsky and McCracken, 1997). This function ensures that only after secretory proteins have folded correctly are they packaged into ER-to-Golgi transport vesicles and permitted to leave the ER. Proteins that fail to fold or assemble correctly are recognized as misfolded and are exported instead, via the Sec61p translocon (Wiertz *et al.*, 1996; Pilon *et al.*, 1997; Plemper *et al.*, 1997), across the ER membrane to the cytosol, where they are degraded by the proteasomes [ER-associated protein degradation (ERAD); Brodsky and McCracken, 1997]. It has been proposed that the toxins translocating into the cytosol from the ER lumen may do so by masquerading as substrates for ERAD (Lord, 1996; Hazes and Read, 1997). This might be achieved after a partial unfolding of the toxin polypeptide, possibly with the assistance of resident ER chaperones. Such unfolding could conceivably lead to translocation competence and recognition as an ERAD

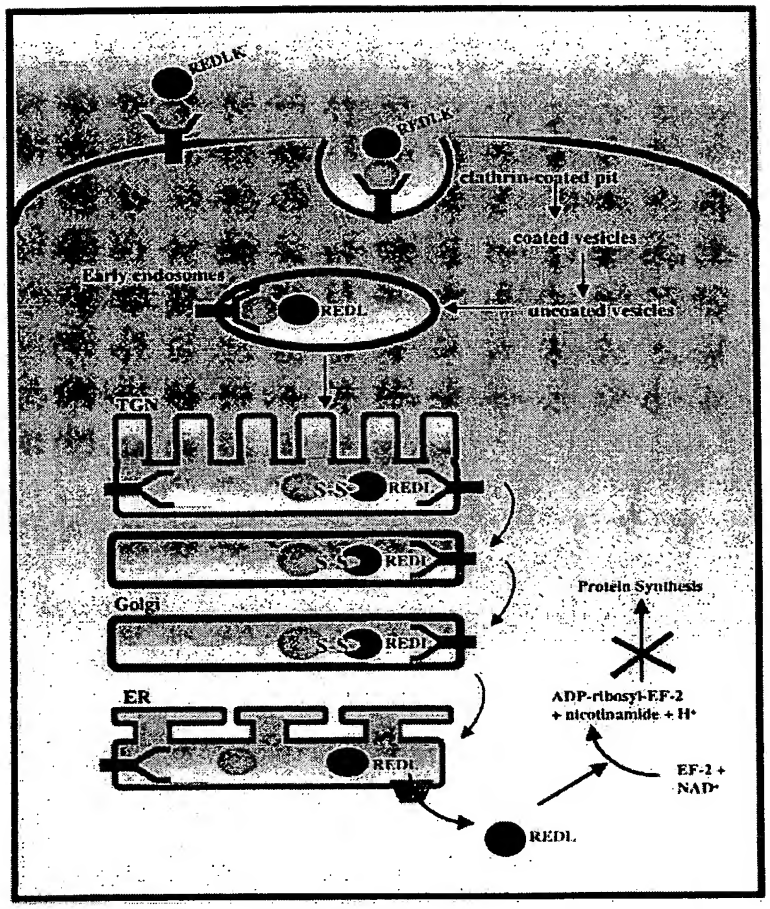

Fig. 2. Toxin entry into mammalian cells from the lumen of the endoplasmic reticulum. PE reaches early endosomes by receptor-mediated endocytosis, as in the case of DT (Fig. 1). PE does not respond to low pH by forming a translocation pore in the endosomal membrane, but undergoes vesicular transport to the TGN. These early steps are accompanied by carboxypeptidase removal of the C-terminal lysine residue and cleavage by furin to generate the disulphide-linked heterodimeric A-B form of the toxin. In the TGN, the receptor-binding domain of PE (yellow) dissociates from its surface receptor, allowing the catalytic domain (red) to interact with the KDEL receptor by virtue of its C-terminal ER-retrieval sequence -REDL. (PE actually folds into three domains. The central domain contains the furin cleavage site, and its function, if any, is not known. For simplicity, it has not been shown here.) The KDEL receptor–PE complex then undergoes retrograde vesicular transport to reach the lumen of the ER, where the receptor-binding and catalytic domains are released from each other by thiol exchange. The catalytic domain is then exported from the ER to the cytosol, probably via the Sec61p translocons normally involved in importing nascent secretory or membrane proteins into the ER. In the cytosol, the catalytic domain inhibits protein synthesis using the same mechanism as that employed by DT. Other toxins that translocate from the ER, such as ST, lack a C-terminal ER-retrieval sequence and must therefore use an alternative to the KDEL receptor as the retrograde carrier for TGN-to-ER transport.

substrate. The mechanism involved in the perception and export of normal ERAD substrates is largely unknown at the present time, and the ways by which bacterial toxins might subvert this system to escape into the cytosol are even less clear. In the cytosol, it is important that at least a fraction of translocated toxin avoids proteasomal degradation and refolds into its catalytically active conformation in order to modify the cytosolic substrate. While the ERAD pathway offers a putative mechanism for toxin export from the ER to the cytosol, the possibility that certain bacterial toxins contain their own translocation apparatus, which somehow becomes activated in the ER lumen, cannot be discounted at present.

It is believed that only the catalytically active A chain of the toxin is translocated into the cytosol. As the A chain is presumably transported to the ER lumen as part of the holotoxin, still disulphide-linked to its B chain, reductive separation of the A and B chains must occur, even within the oxidizing environment of the ER lumen that normally favours disulphide bond formation. This has been demonstrated in the case of CT (Majoul *et al.*, 1997), and disulphide bond reduction in an oxidizing environment was mediated by the resident ER lumen thiol–disulphide exchanger, protein disulphide isomerase (Orlandi, 1997). This is probably also the case for all other disulphide-linked toxins that translocate from the ER.

Conclusions

Bacterial protein toxins that have to cross a membrane in order to encounter their target substrates in mammalian cells use one of two strategies: (i) make a protein-conducting channel through which to pass; or (ii) find a pre-existing protein-conducting channel. After entering cells by endocytosis, those toxins that make a translocation pore do so in early endosomes using a mechanism that depends crucially on the low pH environment within this organelle. Proteins unable to respond to low pH in this way must undergo

retrograde vesicular transport via the TGN and the Golgi stack to reach the lumen of the ER, where they use pre-existing translocation channels, most probably the Sec61p translocon. In the ER lumen, the toxins may partially unfold, possibly with the assistance of ER chaperones, to be perceived as misfolded proteins and as candidates for ERAD. Export from the ER ensues, and at least a proportion of the translocated toxin must refold into its catalytically active native conformation. While retrograde transport of endocytosed toxin to the ER lumen is very inefficient, this is counterbalanced by the extreme potency of the toxins and the amplification that results from their catalytic mode of action. The secretory pathway of eukaryotic cells is therefore completely reversible, a fact that bacterial toxins recognized long before we did.

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